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Attorney Docket No.: 4216.260-US

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

FILING UNDER 37 C.F.R. 1.53(b)

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Assistant Commissioner for Patents
Washington, DC 20231

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Sir:

This is a request for filing a **continuation** application under 37 C.F.R. 1.53(b)

of

Applicant(s): Royer et al.

Title: Non-Toxic, Non-Toxigenic, Non-Pathogenic Fusarium Expression
System

22 pages of specification 16 sheets of drawings

3 sheets of Declaration and Power of Attorney

[x] The filing fee is calculated as follows:

Basic Fee:	\$760.00
Total Claims: 2 - 20 = 0 x 18 =	\$0
Independent Claims: 2 - 3 = 0 x 78 =	\$0
Total Fee:	\$760.00

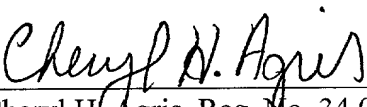
The benefit of application nos. 08/269,449, 08/404,678 and 08/726,105 filed on June 30, 1994, March 15, 1995 and October 4, 1996 in the U.S. is claimed under 35 U.S.C. 120.

Address all future communications to Steve T. Zelson, Esq., Novo Nordisk of North America, Inc., 405 Lexington Avenue, Suite 6400, New York, NY 10174-6401.

Please charge the required fee, estimated to be \$760, to Novo Nordisk of North America, Inc., Deposit Account No. 14-1447. A duplicate of this sheet is enclosed.

Respectfully submitted,

Date: December 15, 1999


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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: Royer et al.

Serial No.: Continuation of appln. Ser. No. Group Art Unit: 1636
08/816,915

Filed: August 29, 1997

Examiner: R. Yucel

For: NON-TOXIC, NON-TOXIGENIC, NON-PATHOGENIC *FUSARIUM* EXPRESSION
SYSTEM

PRELIMINARY AMENDMENT

Please amend the application as follows:

IN THE CLAIMS

Please cancel claims 1-19 without prejudice.

Please add new claims 21-22:

21. (new) A n recombinant host cell having the identifying characteristics of a non-toxic, non-toxigenic, non-pathogenic *Fusarium venenatum* host cell of the section Discolor or a teleomorph or synonym thereof which expresses a heterologous protein, comprising a nucleic acid sequence encoding said heterologous protein operably linked to a promoter.

22. (new) An isolated *Fusarium venenatum* host cell comprising a nucleic acid sequence encoding a heterologous protein.

Please amend claim 20 as follows:

Please change the dependency from "claim 1" to --claim 20--.

REMARKS

Applicants have canceled claims 1-19 and have added new claims 21-22 to recite

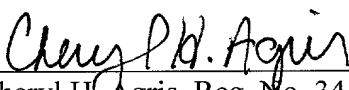
specific embodiments. New claims 21-22 are supported by the specification. Claim 20 has been amended so that it is now dependent on claim 21.

In view of the above, it is respectfully submitted that all claims are in condition for examination. Early action to that end is respectfully requested. The Examiner is hereby invited to contact Steve T. Zelson by telephone at (212) 867-0123 if there are any questions concerning this amendment or application.

Respectfully submitted,

Date:

12/15/99



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**NON-TOXIC, NON-TOXIGENIC, NON-PATHOGENIC
FUSARIUM EXPRESSION SYSTEM**

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This application is a continuation-in-part of Serial No. 08/726,105, filed October 4, 1996, which is a continuation-in-part of of Serial No. 08/404,678 filed March 15, 1995, which is a continuation-in-part of Serial No. 08/269,449 filed June 30, 1994, which are incorporated herein by reference.

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BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates to host cells useful in the production of recombinant proteins. In particular, the invention relates to non-toxic, non-toxigenic, and non-pathogenic fungal host cells of *Fusarium* which can be used in the high-level expression of recombinant proteins, especially enzymes. The invention further relates to promoter and terminator sequences which may be used in such a system.

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Description of the Related Art

The use of recombinant host cells in the expression of heterologous proteins has in recent years greatly simplified the production of large quantities of commercially valuable proteins, which otherwise are obtainable only by purification from their native sources. Currently, there is a varied selection of expression systems from which to choose for the production of any given protein, including prokaryotic and eukaryotic hosts. The selection of an appropriate expression system will often depend not only on the ability of the host cell to produce adequate yields of the protein in an active state, but also to a large extent may be governed by the intended end use of the protein.

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Although mammalian and yeast cells have been the most commonly used eukaryotic hosts, filamentous fungi have now begun to be recognized as very useful as host cells for recombinant protein production. Examples of filamentous fungi which are currently used or

proposed for use in such processes are *Neurospora crassa*, *Acremonium chrysogenum*, *Tolyocladium geodes*, *Mucor circinelloides* and *Trichoderma reesei*, *Aspergillus nidulans*, *Aspergillus niger* and *Aspergillus oryzae*.

Certain species of the genus *Fusarium* have been used as model systems for the studies of plant pathogenicity and gene regulation such as *Fusarium oxysporum* (Diolez et al., 1993, Gene 131:61-67; Langin et al., 1990, Curr. Genet. 17:313-319; Malardier et al., 1989, Gene 78:147-156 and Kistler and Benny, 1988, Curr. Genet. 13:145-149), *Fusarium solani* (Crowhurst et al., 1992, Curr. Genet. 21:463-469), and *Fusarium culmorum* (Curragh et al., 1992, Mycol. Res. 97:313-317). These *Fusarium* sp. would not be suitable commercially for the production of heterologous proteins because of their undesirable characteristics such as being plant pathogens or because they produce unsafe levels of mycotoxin. Dickman and Leslie (1992, Mol. Gen. Genet. 235:458-462) discloses the transformation of *Gibberella zeae* with a plasmid containing *nit-2* of *Neurospora crassa*. The strain of *Gibberella zeae* disclosed in Dickman and Leslie is a plant pathogen and produces zearalenone, an estrogenic mycotoxin. Sanchez-Fernandez et al. (1991, Mol. Gen. Genet. 225:231-233) discloses the transformation of *Gibberella fujikuroi* carrying a *niaD* mutation with a plasmid containing the *Aspergillus niger niaD* gene.

An ideal expression system is one which is substantially free of protease and mycotoxin production, also substantially free of large amounts of other endogenously made secreted proteins, and which is capable of higher levels of expression than known host cells. The present invention now provides new *Fusarium* expression systems which fulfill these requirements.

SUMMARY OF THE INVENTION

The present invention relates to non-toxic, non-toxicogenic, non-pathogenic recombinant *Fusarium* host cell in the section *Discolor* (also known as the section *Fusarium*) or a synonym or teleomorph thereof, comprising a nucleic acid sequence encoding a heterologous protein operably linked to a promoter. The host cells and methods of the present invention are unexpectedly more efficient in the recombinant production of certain fungal enzymes than are other known fungal species, such as *Aspergillus niger*, *Aspergillus oryzae*, or *Fusarium oxysporum*.

The invention also relates to methods for production of heterologous proteins, comprising culturing a host cell of the present invention under conditions conducive to

expression of the protein, and recovering the protein from the culture. In a preferred embodiment, the protein is a fungal protein, most preferably a fungal enzyme. Using the method of the present invention, at least about 0.5 g heterologous protein/l host cell is produced.

5 The invention further relates to a promoter sequence derived from a gene encoding a *Fusarium oxysporum* trypsin-like protease or a fragment thereof having substantially the same promoter activity as said sequence. The sequence of the promoter is shown in SEQ ID NO:5.

10 Additionally, the invention relates to a terminator sequence derived from a gene encoding a *Fusarium oxysporum* trypsin-like protease or a fragment thereof having substantially the same terminator activity as said sequence. The sequence of the terminator is shown in SEQ ID NO:6.

BRIEF DESCRIPTION OF THE FIGURES

15 Figure 1 shows an SDS-PAGE gel of secreted proteins in *Fusarium graminearum* (lane 1); *Aspergillus niger* (lane 2); and *Aspergillus oryzae* (lane 3). Lane 4 shows molecular weight markers.

Figure 2 shows the results of a protease assay on the following samples: *Aspergillus oryzae* (well 1); *Aspergillus niger* (well 2); *Fusarium graminearum* (well 3); empty well controls (wells 4-6).

20 Figure 3 shows the construction of plasmid pJRoy6.

Figure 4 shows SDS-PAGE analysis of the secretion of a trypsin-like protease (SP387) in a transformant of *F. graminearum* ATCC 20334. Lane 1: molecular size markers; lane 2: blank; lane 3: purified trypsin-like protease protein standard; lane 4: blank; lane 5: *F. graminearum* strain ATCC 20334 untransformed; lane 6: blank; lane 7: *F. graminearum* strain ATCC 20334 transformed with plasmid pJRoy6; lane 8: blank; lane 9: molecular size markers.

Figure 5 shows a restriction map of pJRoy20.

Figure 6 shows a restriction map of pDM151.

Figure 7 shows a restriction map of pDM155.

30 Figures 8A and 8B show the level of expression of CAREZYME® in *Fusarium graminearum* when DSM 151-4 is fermented in *Fusarium graminearum* from 20-160 hrs. Figure 8A shows the results of an assay for CAREZYME®. Figure 8B shows SDS-PAGE analysis of the production of CAREZYME® in said *Fusarium graminearum*. Lane 1: molecular

size markers; lane 2:20 hrs.; lane 3:50 hrs.; lane 4:70 hrs.; lane 5:90 hrs.; lane 6:120 hrs.; lane 7:140 hrs.; lane 8:160 hrs.

Figures 9A and 9B show the level of expression of LIPOLASE® when DSM 155-10 is fermented in *Fusarium graminearum* from 20-160 hrs. Figure 9A shows the results of an assay for LIPOLASE®. Figure 9B shows SDS-PAGE analysis of the production of LIPOLASE® in said *Fusarium graminearum*. Lane 1: molecular size markers; lane 2: 20 hrs.; lane 3: 50 hrs.; lane 4: 60 hrs.; lane 5: 90 hrs.; lane 6: 120 hrs.; lane 7: 140 hrs.; lane 8: 160 hrs.

Figure 10 shows a restriction map of pCaHj418.

Figure 11 shows a restriction map of pDM148.

Figure 12 shows a restriction map of pDM149.

Figure 13 shows a restriction map of pMHan37.

Figure 14 shows a restriction map of pDM154.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to non-toxic, non-toxigenic, non-pathogenic recombinant *Fusarium* host cell in the section *Discolor* (also known as the section *Fusarium*) or a synonym or teleomorph thereof, comprising a nucleic acid sequence encoding a heterologous protein operably linked to a promoter.

The known species in the section *Discolor* include, but are not limited to, *Fusarium bactridioides*, *Fusarium cerealis*, *Fusarium crookwellense*, *Fusarium culmorum*, *Fusarium graminearum*, *Fusarium graminum*, *Fusarium heterosporum*, *Fusarium negundi*, *Fusarium reticulatum*, *Fusarium roseum*, *Fusarium sambucinum*, *Fusarium sarcochroum*, *Fusarium sulphureum*, *Fusarium trichothecioides*, *Fusarium venenatum*, most specifically, *Fusarium venenatum* Nirenberg sp. nov., and *Fusarium toruloseum*. Known teleomorphs of *Fusarium* of the section *Discolor* include, but are not limited to, *Gibberella gordonii*, *Gibberella cyanea*, *Gibberella pulicaris*, and *Gibberella zeae*.

Fusarium strains are characterized by mycelium extensive and cotton-like in culture, often with some tinge of pink, purple or yellow in the mycelium on solid medium. Conidiophores are variable slender and simple, or stout, short, branched irregularly or bearing a whorl of phialides, single or grouped into sporodochia. Conidia are principally of two kinds, often held in small moist heads: macroconidia several-celled, slightly curved or bent at the pointed ends, typically canoe-shaped and microconidia which are one celled, ovoid or oblong,

borne singly or in chains. Some conidia are intermediate, 2 or 3 celled, oblong or slightly curved.

As defined herein, "non-toxic" means that the host cell does not act as a poison to plants or animals. For example, a *Fusarium* host cell would be considered non-toxic if about 14 days after injecting about 5 mice with a dose of about 20 ml of (1:1 diluted) 3 day old *Fusarium* culture medium/kg body wt./mouse, none of the mice died as a result of *Fusarium* treatment. As defined herein, "non-toxigenic" means that the host cells are essentially free of mycotoxin as determined by standard analytical methods such as HPLC analysis. For example, an amount of *Fusarium* grown on 2 x 9 cm petri dishes containing solid nutrient medium may be extracted with organic solvents and 0.5% of the extract may be injected into an HPLC for analysis. The absence of known mycotoxins would be inferred by the absence of detectable HPLC peaks at positions known for mycotoxin standards. As defined herein, "non-pathogenic" means that the host cells do not cause significant disease in healthy plants or healthy animals. For example, a *Fusarium* sp. that is pathogenic to plants can show a fungal invasion of the xylem tissue of the plant and result in the disease state characterized by typical wilt symptoms. As defined herein, a "heterologous protein" is a protein which is not native to the host cell, or a native protein in which modifications have been made to alter the native sequence or a native protein whose expression is quantitatively altered as a result of a manipulation of a native regulatory sequence required for the expression of the native protein, such as a promoter, a ribosome binding site, etc. or other manipulation of the host cell by recombinant DNA techniques. The nucleic acid sequence is operably linked to a suitable promoter sequence, which is capable of directing transcription of the nucleic acid sequence in the chosen host cell.

In a specific embodiment, the host cells of the present invention are of the species *Fusarium graminearum* which is characterized by the following features. Conidia: Microconidia are absent. Macroconidia are distinctly septate, thick walled, straight to moderately sickle-shaped, unequally curved with the ventral surface almost straight and a smoothly arched dorsal surface. The basal cell is distinctly foot-shaped. The apical cell is cone-shaped or constricted as a snout. Conidiophores: unbranched and branched monophialides. Chlamydospores: are generally very slow to form in culture: when they do occur, they most often form in the macroconidia but may also form in the mycelium. Colony morphology: on PDA, growth is rapid with dense aerial mycelium that may almost fill the tube and is frequently yellow to tan with the margins white to carmine red. Red-brown to orange

sporodochia, if present, are sparse, often appearing only when the cultures are more than 30 days old. The undersurface is usually carmine red. This fungus produces the most cylindrical (dorsal and ventral surfaces parallel) macroconidia of any species of the section Discolor.

In a most specific embodiment, the *Fusarium* strain has been deposited with the American Type Culture Collection and assigned the number ATCC 20334 and has been identified as *Fusarium graminearum* Schwabe IMI 145425 in U.S. Patent No. 4,041,189. The *Fusarium* strain may also be derivatives and mutants which are similarly non-toxic, non-toxigenic, and non-pathogenic, e.g. those taught in U.S. Patent No. 4,041,189.

It will be understood that throughout the specification and claims the use of the term "*Fusarium graminearum*" refers not only to organisms encompassed in this species, but also includes those species which have previously been or currently are designated as other species in alternate classification schemes, but which possess the same morphological and cultural characteristics defined above, and may be synonymous to *F. graminearum*. These include but are not limited to *Fusarium roseum*, *F. roseum* var. *graminearum*, *Gibberella zeae*, or *Gibberella roseum*, *Gibberella roseum* f. sp. *cerealis*.

The skilled artisan will also recognize that the successful transformation of the host species described herein is not limited to the use of the vectors, promoters, and selection markers specifically exemplified. Generally speaking, those techniques which are useful in transformation of *F. oxysporum*, *F. solani* and *F. culmorum* are also useful with the host cells of the present invention. For example, although the *amdS* selection marker is preferred, other useful selection markers include the *argB* (*A. nidulans* or *A. niger*), *trpC* (*A. niger* or *A. nidulans*), *pyrG* (*A. niger*, *A. oryzae* or *A. nidulans*), *niaD* (*A. nidulans*, *A. niger*, or *F. oxysporum*), and *hygB* (*E. coli*) markers. The promoter may be any DNA sequence that shows strong transcriptional activity in these species, and may be derived from genes encoding both extracellular and intracellular proteins, such as amylases, glucoamylases, proteases, lipases, cellulases and glycolytic enzymes. Examples of such promoters include but are not limited to *A. nidulans amdS* promoter or promoters from genes for glycolytic enzymes, e.g., TPI, ADH, GAPDH, and PGK. The promoter may also be a homologous promoter, i.e., the promoter for a gene native to the host strain being used. The promoter sequence may also be provided with linkers for the purpose of introducing specific restriction sites facilitating ligation of the promoter sequence with the gene of choice or with a selected signal peptide or preregion.

The promoter sequence may be derived from a gene encoding a *Fusarium oxysporum* trypsin-like protease or a fragment thereof having substantially the same promoter activity as

said sequence. The sequence of the promoter is shown in SEQ ID NO:5. The invention further encompasses nucleic acid sequences which hybridize to the promoter sequence shown in SEQ ID NO:5 under the following conditions: presoaking in 5X SSC and prehybridizing for 1 hr. at about 40°C in a solution of 20% formamide, 5X Denhardt's solution, 50 mM sodium phosphate, pH 6.8, and 50 ug denatured sonicated calf thymus DNA, followed by hybridization in the same solution supplemented with 100 uM ATP for 18 hrs. at about 40°C, followed by a wash in 0.4X SSC at a temperature of about 45°C, or which have at least about 90% homology and preferably about 95% homology to SEQ ID NO:5, but which have substantially the same promoter activity as said sequence. In another embodiment, the promoter may be a sequence comprising a large number of binding sites of AreA, a positive regulator of genes expressed during nitrogen limitation; these sites are referred to as *nit-2* in *Neurospora crassa* (Fu and Marzlus, 1990, Proc. Natl. Acad. Sci. U.S.A. 87:5331-5335). The promoter sequence may be modified by the addition or substitution of such AreA sites.

Terminators and polyadenylation sequences may also be derived from the same sources as the promoters. In a specific embodiment, the terminator sequence may be derived from a gene encoding a *Fusarium oxysporum* trypsin-like protease or a fragment thereof having substantially the same terminator activity as said sequence. The sequence of the terminator is shown in SEQ ID NO:6. The invention further encompasses nucleic acid sequences which hybridize to the terminator sequence shown in SEQ ID NO:6 under the following conditions: presoaking in 5X SSC and prehybridizing for 1 hr. at about 40°C in a solution of 20% formamide, 5X Denhardt's solution, 50 mM sodium phosphate, pH 6.8, and 50 ug denatured sonicated calf thymus DNA, followed by hybridization in the same solution supplemented with 100 uM ATP for 18 hrs. at about 40°C, followed by a wash in 0.4X SSC at a temperature of about 45°C, or which have at least about 90% homology and preferably about 95% homology to SEQ ID NO:5, but which have substantially the same terminator activity as said sequence.

Enhancer sequences may also be inserted into the construct.

To avoid the necessity of disrupting the cell to obtain the expressed product, and to minimize the amount of possible degradation of the expressed product within the cell, it is preferred that the product be secreted outside the cell. To this end, in a preferred embodiment, the gene of interest is linked to a preregion such as a signal or leader peptide which can direct the expressed product into the cell's secretory pathway. The preregion may be derived from genes for any secreted protein from any organism, or may be the native preregion. Among

useful available sources for such a preregion are a glucoamylase or an amylase gene from an *Aspergillus* species, an amylase gene from a *Bacillus* species, a lipase or proteinase gene from *Rhizomucor miehei*, the gene for the alpha-factor from *Saccharomyces cerevisiae*, or the calf prochymosin gene. The preregion may be derived from the gene for *A. oryzae* TAKA amylase, *A. niger* neutral alpha-amylase, *A. niger* acid stable α -amylase, *B. licheniformis* α -amylase, the maltogenic amylase from *Bacillus* NCIB 11837, *B. stearothermophilus* α -amylase, or *B. licheniformis* subtilisin. An effective signal sequence is the *A. oryzae* TAKA amylase signal, the *Rhizomucor miehei* aspartic proteinase signal and the *Rhizomucor miehei* lipase signal. As an alternative, the preregion native to the gene being expressed may also be used, e.g., in SEQ ID NO:4 between amino acids -24 and -5.

The gene for the desired product functionally linked to promoter and terminator sequences may be incorporated in a vector containing the selection marker or may be placed on a separate vector or plasmid capable of being integrated into the genome of the host strain. Alternatively, the vectors used may be capable of replicating as linear or circular extrachromosomal elements in the host cell. These types of vectors include for example, plasmids and minichromosomes. The vector system may be a single vector or plasmid or two or more vectors or plasmids which together contain the total DNA to be integrated into the genome. Vectors or plasmids may be linear or closed circular molecules.

The host cell may be transformed with the nucleic acid encoding the heterologous protein using procedures known in the art such as transformation and electroporation (see, for example, Fincham, 1989, Microbial Rev. 53:148-170).

The recombinant host cell of the present invention may be cultured using procedures known in the art. Briefly, the host cells are cultured on standard growth medium such as those containing a combination of inorganic salts, vitamins, a suitable organic carbon source such as glucose or starch, any of a variety of complex nutrients sources (yeast extract, hydrolyzed casein, soya bean meal, etc.). One example is FP-1 medium (5% soya bean meal, 5% glucose, 2% K_2HPO_4 , 0.2% $CaCl_2$, 0.2% $MgSO_4 \cdot 7H_2O$ and 0.1% pluronic acid (BASF)). The fermentation is carried out at a pH of about 4.5-8.0, and at a temperature of about 20-37°C for about 2-7 days.

The present host cell species can be used to express any prokaryotic or eukaryotic heterologous protein of interest, and is preferably used to express eukaryotic proteins. Of particular interest for these species is their use in expression of heterologous proteins, especially fungal enzymes. The novel expression systems can be used to express enzymes such

as catalase, laccase, phenoloxidase, oxidase, oxidoreductases, cellulase, xylanase, peroxidase, lipase, hydrolase, esterase, cutinase, protease and other proteolytic enzymes, aminopeptidase, carboxypeptidase, phytase, lyase, pectinase and other pectinolytic enzymes, amylase, glucoamylase, alpha-galactosidase, beta-galactosidase, alpha-glucosidase, beta-glucosidase, mannosidase, isomerase, invertase, transferase, ribonuclease, chitinase, mutanase and deoxyribonuclease.

In a specific embodiment, the enzyme is an alkaline protease, e.g., a *Fusarium oxysporum* pre-pro-trypsin gene. In a most specific embodiment, the genomic sequence is shown in SEQ ID NO:3 and the protein sequence is shown in SEQ ID NO:4.

In another specific embodiment, the enzyme is an alkaline endoglucanase, which is immunologically reactive with an antibody raised against a highly purified ~43 kD endoglucanase derived from *Humicola insolens*, DSM 1800, or which is a derivative of the ~43 kD endoglucanase exhibiting cellulase activity (cf. WO 91/17243). The endoglucanase, hereinafter referred to as "CAREZYME®" may be encoded by a gene shown in SEQ ID NO:7 and may have a protein sequence shown in SEQ ID NO:8. The enzyme may also be a CAREZYME® variant.

In yet another specific embodiment, the enzyme is a 1,3-specific lipase, hereinafter referred to as LIPOLASE®. The enzyme may be encoded by the DNA sequence shown in SEQ ID NO:9 and may have an amino acid sequence shown in SEQ ID NO:10. The enzyme may also be a LIPOLASE® variant, e.g., D96L, E210K, E210L (see WO 92/05249).

It will be understood by those skilled in the art that the term "fungal enzymes" includes not only native fungal enzymes, but also those fungal enzymes which have been modified by amino acid substitutions, deletions, additions, or other modifications which may be made to enhance activity, thermostability, pH tolerance and the like. The present host cell species can also be used to express heterologous proteins of pharmaceutical interest such as hormones, growth factors, receptors, and the like.

The present invention will be further illustrated by the following non-limiting examples.

EXAMPLES

Example 1 - *Fusarium graminearum* ATCC 20334 Secretes Only a Low Level of Protein

Conidial spore suspensions of *Fusarium graminearum* strain ATCC 20334, an *A. oryzae*, and *A. niger* are inoculated into 25 ml of YPD medium (1 % yeast extract (Difco), 2 % bactopectone (Difco), 2 % glucose) in a 125 ml shake flask and incubated at 30°C at 300 rpm

for 5 days. Supernatant broths from the cultures are harvested by centrifugation. A total of 10 μ l of each sample are mixed with 10 μ l 0.1 M dithiothreitol (Sigma) and 10 μ l of loading buffer (40 mM Tris base, 6% sodium dodecyl sulfate, 2.5 mM EDTA, 15% glycerol, 2 mg/ml bromocresol purple). The samples are boiled for 5 minutes and run on a 4-12% polyacrylamide gel (Novex). The proteins are visualized by staining with Coomassie Blue. The results (Figure 1) show that *Fusarium graminearum* strain ATCC 20334 produces very little secreted protein.

Example 2 - *Fusarium graminearum* ATCC 20334 Secretes Only a Low Level of Proteases

A total of 40 μ l of culture broths from *Fusarium graminearum* strain ATCC 20334, *A. oryzae*, and *A. niger* (see Example 1) are each pipetted into wells that are cut into a casein agar plate (2% non-fat dry milk (Lucerne), 50 mM Tris-HCl pH=7.5, 1% noble agar (Difco)). The plates are incubated at 37°C for 5 hours and the zones of protein hydrolysis are observed. The results (Figure 2) show that *Fusarium graminearum* strain ATCC 20334 broth contains very little proteolytic activity.

Example 3 - Cloning of *Fusarium oxysporum* Genomic Prepro-trypsin Gene

A genomic DNA library in lambda phage is prepared from the *F. oxysporum* genomic DNA using methods such as those described found in Sambrook *et al.*, 1989, Molecular Cloning, A Laboratory Manual, Cold Spring Harbor, NY. A total of 50 μ g genomic DNA are digested in a volume of 200 μ l containing 10 mM Tris (pH=7.5), 50 mM NaCl, 7 mM MgCl₂, 7 mM 2-mercaptoethanol, and 4 units restriction enzyme Sau3A for one minute at 37°C. Partially digested DNA of molecular size 10-20 kb is isolated by agarose gel electrophoresis, followed by electroelution into dialysis membrane and concentration using an Elutip-D column (Schleicher and Schuell). One μ g of lambda arms of phage of EMBL4 that had been cut with restriction enzyme *Bam*H1 and treated with phosphatase (Clontech) is ligated with 300-400 μ g Sau3A cut genomic DNA in a volume of 25 μ l under standard conditions (see Sambrook *et al.*, 1989, Molecular Cloning, A Laboratory Manual, Cold Spring Harbor, NY). Lambda phage are prepared from this ligation mix using a commercially available kit (Gigapack Gold II, Stratagene) following the manufacturers directions.

The plating of ca. 15,000 recombinant lambda phage and the production of filter lifts (to Hybond N⁺ filters, Amersham) are performed using standard methods (Sambrook *et al.*, 1989, Molecular Cloning, A Laboratory Manual, Cold Spring Harbor, NY). The filters are processed for hybridization with a Genius Kit for nonradioactive nucleic acids detection

(Boehringer Mannheim) using standard methods (Sambrook *et al.*, 1989, Molecular Cloning, A Laboratory Manual, Cold Spring Harbor, NY). The DNA used as a probe is a 0.75 kb digoxigenin (DIG) labeled PCR fragment of the entire coding region of the *F. oxysporum* trypsin-like protease (hereinafter referred to as SP387) gene present in plasmid pSX233, which has been deposited with the NRRL under the accession number of NRRL B-21241. The primers for the PCR reaction are 5'-tgcg gatccATGGTCAAGTTCGCTTCCGTC (forward primer; SEQ ID NO:1) and 5'-gacctcgagTTAAGCATAGGTGTCAATGAA (reverse primer; SEQ ID NO:2). In both primers, the lower case characters represent linker sequences and the upper case characters correspond to the coding region of the SP387 gene. To perform the PCR, 25 ng of a 907 bp BamH1/Xba1 DNA fragment containing the SP387 gene from plasmid pSX233 are mixed with 68 pmoles of each forward and reverse primer.

The mixture of the DNA fragment and primers is made up to an 80 μ l volume in 1X Taq Buffer/1X DIG labelling Mix/5 units Taq (Boehringer Mannheim). The reaction conditions are 95°C, 3 minutes, then 35 cycles of [95°C 30 seconds, 50°C 1 minute, 72°C 1 minute]. The DNA sequence derived by PCR from the *F. oxysporum* trypsin-like protease is shown in SEQ ID NO:3. The phage plaques are screened with the DIG labeled probe using a modification (Engler and Blum, 1993, Anal. Biochem. 210:235-244) of the Genius kit (Boehringer Mannheim). Positive clones are isolated and purified by a second round of plating and hybridization. Recombinant lambda phage containing the *F. oxysporum* trypsin-like protease gene are prepared and DNA is isolated from the phage using a Quiagen lambda midi preparation kit (Quiagen).

Example 4 - Construction of Expression Plasmid pJRoy6

Restriction mapping, Southern blotting, and hybridization techniques (Sambrook *et al.*, 1989, Molecular Cloning, A Laboratory Manual, Cold Spring Harbor, NY) are used to identify a 5.5 kb Pst1 restriction enzyme fragment from one of the recombinant phage that contains the *F. oxysporum* trypsin-like protease coding gene and flanking DNA sequences. This 5.5 kb Pst1 fragment is subcloned into Pst1 digested pUC118 and the plasmid is designated pJRoy4 (see Figure 3). Plasmid pJRoy4 is digested with restriction enzyme EcoR1 and a 3.5 kb EcoR1 fragment containing the SP387 gene and the 43 bp EcoR1/Pst1 region of the pUC118 polylinker is isolated and subcloned into the vector pToC90 to create plasmid pJRoy6 (Figure 3).

Example 5 - Construction of SP387 Expression Cassette

An expression cassette (pJRoy20) containing the SP387 promoter and terminator joined by a BamH1 site in pUC118 is constructed. An *E. coli* strain containing pJRoy20 has been deposited with the NRRL. The promoter fragment is generated by digesting the SP387 vector pJRoy6 with EcoR1 (which cuts at -1200) and with Nco1 (which cuts at the translational start site, see Figure 5). The terminator sequence (bp 2056-3107 in Figure 5) is generated by PCR amplification using the following oligonucleotides:

FORWARD

5'gcacaccatggctcgctggatccATACCTTGTTGGAAGCGTCG3' (SEQ ID NO:11)

REVERSE

5'atcggagcatgcggtaccgtttaacgaattcAGGTAAACAAGATATAATTTTCTG 3' (SEQ ID NO:12)

Letters in large case are complementary to SP387 terminator DNA, while lower case letters are tails containing engineered restriction sites.

After digestion with Nco1 and Sph1, the resulting amplification product containing the terminator flanked by Nco1 and BamH1 sites on the 5' end, and flanked by EcoR1, Pme1, Kpn1 and Sph1 sites on the 3' end is isolated. A 3-way ligation between the promoter fragment, the terminator fragment and Kpn1/Sph1 cut pUC118 is performed to generate pJRoy20 (see Figure 5).

Example 6 - CAREZYME® Constructs

The EcoRV site at -15 in the SP387 promoter, and the Nco1 site present at +243 in the CAREZYME® coding region are utilized to create an exact fusion between the SP387 promoter and the CAREZYME® gene. A PCR fragment containing -18 to -1 of the SP387 promoter directly followed by -1 to + 294 of the CAREZYME® gene is generated from the CAREZYME® vector pCaHj418 (see Figure 10) using the following primers:

FORWARD

EcoRV

5'ctcttgatatctatctcttcaccATGCGTTCCTCCCCCTCCT3' (SEQ ID NO:13)

REVERSE

5'CAATAGAGGTGGCAGCAAAA 3' (SEQ ID NO:14)

Lower case letters in the forward primer are bp -24 to -1 of the SP387 promoter, while upper case letters are bp 1 to 20 of CAREZYME®.

The PCR conditions used are: 95°C, 5 min. followed by 30 cycles of [95°C, 30 sec., 50°C, 1 min., 72°C, 1 min.]. The resulting 0.32 kb fragment is cloned into vector pCRII using Invitrogen's TA cloning kit resulting in pDM148 (see Figure 11). The 0.26 kb EcoRV/NcoI fragment is isolated from pDM148 and ligated to the 0.69 kb NcoI/BglII fragment from pCaHj418 and cloned into EcoRV/BamHI digested pJRoy20 to create pDM149 (see Figure 12). The 3.2 kb EcoRI CAREZYME® expression cassette (SP387 promoter/CAREZYME®/SP387 terminator) is isolated from pDM149 and cloned into the EcoRI site of pToC90 to create pDM151 (see Figure 6). Expression construct pDM151 contains both the expression cassette and the *amdS* selectable marker. An *E. coli* strain containing pDM151 has been deposited with the NRRL.

Example 7 - LIPOLASE® Constructs

The EcoRV site at -15 in the SP387 promoter, and the SacI site at +6 in the LIPOLASE® coding region are utilized to create an exact fusion between the SP387 promoter and the LIPOLASE® gene. An adapter containing the final 15 bp of the SP387 promoter followed by the first 6 bp of the LIPOLASE® coding region is constructed and is shown below.

EcoRV	SacI
atctatctcttcaccATGAGGAGCT	(SEQ ID NO:15)
tagatagagaagtggTACTCC	(SEQ ID NO:16)

A 0.9 kb SacI/BamHI fragment of the LIPOLASE® cDNA gene is isolated from the *A. oryzae* expression construct pMHan37 (see Figure 13). The EcoRV/SacI adapter and SacI/BamHI LIPOLASE® fragment are ligated and cloned into EcoRV/BamHI digested pJRoy20 to create plasmid pDM154 (see Figure 14). The 3.2 kb KpnI LIPOLASE® expression cassette (SP387 promoter/LIPOLASE®/SP387 terminator) is isolated from pDM154 and cloned into the KpnI site of pToC90 to create plasmid pDM155 (see Figure 7). Expression construct pDM155

contains both the LIPOLASE® expression cassette and the *amdS* selectable marker. An *E. coli* strain containing pDM151 has been deposited with the NRRL.

Example 8 - Transformation of *F. graminearum*

5 *Fusarium graminearum* strain ATCC 20334 cultures are grown on 100 x 15 mm petri plates of Vogels medium (Vogel, 1964, Am. Nature 98:435-446) plus 1.5% glucose and 1.5% agar for 3 weeks at 25°C. Conidia (approximately 10⁸ per plate) are dislodged in 10 ml of sterile water using a transfer loop and purified by filtration through 4 layers of cheesecloth and finally through one layer of miracloth. Conidial suspensions are concentrated by
10 centrifugation. Fifty ml of YPG (1% yeast extract (Difco) 2% bactopectone (Difco), 2% glucose) are inoculated with 10⁸ conidia, and incubated for 14 h at 20°C, 150 rpm. Resulting hyphae are trapped on a sterile 0.4 µm filter and washed successively with sterile distilled water and 1.0 M MgSO₄. The hyphae are resuspended in 10 ml of Novozym® 234 (Novo Nordisk) solution (2-10 mg/ml in 1.0 M MgSO₄) and digested for 15-30 min at 34°C
15 with agitation at 80 rpm. Undigested hyphal material is removed from the resulting protoplast suspension by successive filtration through 4 layers of cheesecloth and through miracloth. Twenty ml of 1M sorbitol are passed through the cheesecloth and miracloth and combined with the protoplast solution. After mixing, protoplasts (approximately 5 x 10⁸) are pelleted by centrifugation and washed successively by resuspension and centrifugation in 20 ml of 1M
20 sorbitol and in 20 ml of STC (0.8 M sorbitol, 50 mM Tris-HCl pH=8.0, 50 mM CaCl₂). The washed protoplasts are resuspended in 4 parts STC and 1 part SPTC (0.8M sorbitol, 40% polyethylene glycol 4000 (BDH), 50 mM Tris-HCl pH=8.0, 50 mM CaCl₂) at a concentration of 1-2 x 10⁸/ml. One hundred µl of protoplast suspension are added to 5 µg pJRoy6 and 5 µl heparin (5 mg/ml in STC) in polypropylene tubes (17 x 100 mm) and
25 incubated on ice for 30 min. One ml of SPTC is mixed gently into the protoplast suspension and incubation is continued at room temperature for 20 min. Protoplasts are plated on a selective medium consisting of Cove salts (Cove, D.J., 1966, Biochem. Biophys. Acta 113:51-56) plus 10 mM acetamide, 15 mM CsCl₂, 2.5% noble agar (Difco) and 1.0 M sucrose using an overlay of the same medium with 0.6 M sucrose and 1.0% low melting agarose (Sigma).
30 Plates are incubated at 25°C and transformants appeared in 6-21 days.

Example 9 - Expression of trypsin-like protease in *Fusarium graminearum*

Transformants are transferred to plates of COVE2 medium (same as COVE medium above without the cesium chloride and replacing the 1.0 M sucrose with a concentration of 30 g/l) and grown for 3 or more days at 25°C. Twenty five ml aliquots of FP-1 medium (5% soya bean meal, 5% glucose 2% K₂HPO₄, 0.2% CaCl₂, 0.2% MgSO₄·7H₂O and 0.1% pluronic acid (BASF)) in 150 ml flasks are inoculated with approximately 1 cm agar plugs from COVE2 plate cultures and incubated for 6 days at 30°C with agitation (150 rpm). Supernatant broth samples are recovered after centrifugation and subjected to SDS-PAGE analysis as follows. Thirty µl of each broth is mixed with 10 µl SDS-PAGE sample buffer (1 ml 0.5 M Tris pH=6.8, 0.8 ml glycerol, 1.6 ml 10% SDS, 0.4 ml 0.8 M dithiothreitol, 0.2 ml 1% bromophenol blue), 2 µl of 2% PMSF (Sigma) in isopropanol, and 2 µl glycerol. The samples are placed in a boiling water bath for 4 minutes and 40 µl of each are run on a 10-27% polyacrylamide gel (Novex). The gels are stained and destained with Coomassie dye using standard methods. The expression level of the trypsin-like protease has been determined to be ≥ 0.5 g/l.

Example 10 - Enzyme assays

A. CAREZYME®

Buffer: Sodium phosphate (50 mM, pH 7.0)

Substrate: AZCL-HE cellulose (Megazyme) at 2 mg/ml buffer

Enzyme std: 100 mg of CAREZYME® standard (10,070 ECU/g) is dissolved in 1 ml buffer and stored at -20°C. This stock is diluted 1:100 in buffer immediately prior to use in enzyme assays. The assay range is 0.5 - 5.0 ECU/ml. A conversion factor of 650,000 ECU/g CAREZYME® is used.

Substrate solution (990 µl) is added to sample wells of a 24-well microtiter plate. Ten µl of CAREZYME® sample (diluted in buffer to produce activity of between 0.5 and 10 ECU/ml.) are added to the substrate. Reactions are incubated for 30 minutes at 45°C with vigorous shaking, and next centrifuged at 4°C for 5 minutes at 5,000 rpm. Two hundred µl of supernatant are transferred to a 96-well microtiter plate and the absorbance at 650 nm is measured.

B. LIPOLASE® Assay

Buffer: 0.1M MOPS, pH 7.5 containing 4 mM CaCl₂

Substrate: 10 mL p-nitrophenyl butyrate (pNB)

in 1 ml DMSO;

Add 4 ml buffer to substrate in DMSO

*Stock concentration = 11.5 mM in 20% DMSO

Enzyme std: LIPOLASE® (23,100 LU/g) is dissolved at 1000 LU/ml in 50% glycerol and stored at -20°C. This stock is diluted 1:100 in buffer immediately prior to assay. The assay range is 0.125 to 3.0 LU/ml.

100 µl pNB stock solution is added to 100 µl of appropriately diluted enzyme sample. Activity (mOD/min) is measured at 405 nm for 5 min at 25°C.

C. SP387 Assay

L-BAPNA substrate is prepared by dilution of a 0.2 M stock solution of L-BAPNA (Sigma B3133) in dimethyl sulfoxide (stored frozen) to 0.004 M in buffer (0.01 M dimethylglutaric acid (Sigma), 0.2 M boric acid and 0.002 M calcium chloride, adjusted to pH 6.5 with NaOH) just prior to use. One µl of culture was centrifuged (145000 x g, 10 min). A 100 µl aliquot of diluted culture broth is added to 100 µl substrate in a 96 well microtiter plate. Absorption change at 405 nm is assayed at 30 second intervals for 5 min. at 25°C using an ELISA reader. Results are calculated relative to a purified SP387 standard.

Example 11 - Expression of CAREZYME®

Twenty-three transformants of pDM151 are purified, cultured in shake flasks on soy/glucose medium and assayed for CAREZYME® activity after 9 days (Table 1-see below). Four transformants express CAREZYME® at a level of approximately 50-100 mg/L. Transformant pDM151-4 is cultured in small scale fermentors using the conditions developed for SP387 production (see Example 9). Approximately 6.0 g/L of CAREZYME® is evident after 7 days (Figure 8A). CAREZYME® comprised greater than 90% of secreted proteins based on SDS gel electrophoresis (Figure 8B).

TABLE I

Transformant #	EMU/ml	mg/L
pDM 151.3 - 4	58.2	90
pDM 151.3 - 5	0	0

	pDM 151.3 - 6	0	0
	pDM 151.3 - 10	0	0
	pDM 151.3 - 11	2.46	4
	pDM 151.3 - 12	0	0
5	pDM 151.3 - 13	12.2	19
	pDM 151.3 - 14	47.3	73
	pDM 151.3 - 15	22.7	35
	pDM 151.3 - 16	0	0
	pDM 151.3 - 17	0	0
10	pDM 151.3 - 18	0	0
	pDM 151.3 - 19	0	0
	pDM 151.3 - 21	0	0
	pDM 151.3 - 22	43.7	67
	pDM 151.3 - 23	1.25	2
15	pDM 151.3 - 24	17.8	27
	pDM 151.3 - 25	38	58
	pDM 151.3 - 26	0	0
	pDM 151.3 - 27	10.5	16
	pDM 151.3 - 28	49.3	76
20	pDM 151.3 - 29	19.8	30
	pDM 151.3 - 30	22.7	35

Example 12 - Expression of LIPOLASE®

Fifteen transformants of pDM155 are purified, cultured in shake flasks in soy/glucose medium and assayed for LIPOLASE® activity after 9 days (Table 2-see next page).

TABLE II

Transformant #	LU/ml	mg/ml
pDM 155 - 1	669	167
pDM 155 - 2	45.2	11

pDM 155 - 3	180	45
pDM 155 - 4	0	0
pDM 155 - 5	55.4	14
pDM 155 - 6	116	29
pDM 155 - 7	704	176
pDM 155 - 8	214	54
pDM 155 - 9	17.1	4
pDM 155 - 10	712	178
pDM 155 - 11	511	128
pDM 155 - 12	0	0
pDM 155 - 13	0	0
pDM 155 - 14	0	0
pDM 155 - 15	153	38
pDM 155 - 16	0	0
pDM 155 - 17	0	0
pDM 155 - 18	0	0
pDM 155 - 19	129	32
pDM 155 - 20	378	95
pDM 155 - 21	216	54

Four transformants expressed LIPOLASE® at a level of approximately 100-200 mg/l (based on the pNB assay). Transformant pDM155-10 is cultured in small scale fermentors using the conditions developed for SP387 production (see Example 9). Approximately 2.0 g/l of LIPOLASE is evident after 7 days (Figure 8A). LIPOLASE® comprised greater than 90% of secreted proteins based on SDS gel electrophoresis (Figure 8B).

DEPOSIT OF MICROORGANISMS

The following biological materials have been deposited in the Agricultural Research Service Patent Culture Collection (NRRL), Northern Regional Research Center, 1815 University Street, Peoria, Illinois, 61604, USA.

Strain	Accession No.	Deposit Date
<i>E. coli</i> containing pJRoy6	NRRL B-21285	6/20/94
<i>E. coli</i> containing pJRoy20	NRRL B-21418	3/10/95
5 <i>E. coli</i> containing pDM151	NRRL B-21419	3/10/95
<i>E. coli</i> containing pDM155	NRRL B-21420	3/10/95

The strains have been deposited under conditions that assure that access to the culture will be available during the pendency of this patent application to one determined by the Commissioner of Patents and Trademarks to be entitled thereto under 37 C.F.R. §1.14 and 35 U.S.C. §122 and under conditions of the Budapest Treaty. The deposit represents a biologically pure culture of each deposited strain. The deposit is available as required by foreign patent laws in countries wherein counterparts of the subject application, or its progeny are filed. However, it should be understood that the availability of a deposit does not constitute a license to practice the subject invention in derogation of patent rights granted by governmental action.

The invention described and claimed herein is not to be limited in scope by the specific embodiments herein disclosed, since these embodiments are intended as illustrations of several aspects of the invention. Any equivalent embodiments are intended to be within the scope of this invention. Indeed, various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description. Such modifications are also intended to fall within the scope of the appended claims.

Various references are cited herein, the disclosures of which are incorporated by reference in their entireties.

CLAIMS

What is claimed is:

5 1. A non-toxic, non-toxigenic, non-pathogenic recombinant *Fusarium* host cell of the section Discolor or a teleomorph or synonym thereof, comprising a nucleic acid sequence encoding a heterologous protein operably linked to a promoter.

10 2. The host cell of claim 1 in which the *Fusarium* host cell is a *Fusarium bactridioides* cell.

15 3. The host cell of claim 1 in which the *Fusarium* host cell is a *Fusarium cerealis* cell.

20 4. The host cell of claim 1 in which the *Fusarium* host cell is a *Fusarium crookwellense* cell.

25 5. The host cell of claim 1 in which the *Fusarium* host cell is a *Fusarium culmorum* cell.

30 6. The host cell of claim 1 in which the *Fusarium* host cell is a *Fusarium graminearum* cell.

7. The host cell of claim 1 in which the *Fusarium* host cell is a *Fusarium gramineum* cell.

8. The host cell of claim 1 in which the *Fusarium* host cell is a *Fusarium heterosporum* cell.

9. The host cell of claim 1 in which the *Fusarium* host cell is a *Fusarium negundi* cell.

10. The host cell of claim 1 in which the *Fusarium* host cell is a *Fusarium reticulatum* cell.

11. The host cell of claim 1 in which the *Fusarium* host cell is a *Fusarium roseum* cell.

12. The host cell of claim 1 in which the *Fusarium* host cell is a *Fusarium sambucinum* cell.

13. The host cell of claim 1 in which the *Fusarium* host cell is a *Fusarium sarcochroum* cell.

14. The host cell of claim 1 in which the *Fusarium* host cell is a *Fusarium sulphureum* cell.

15. The host cell of claim 1 in which the *Fusarium* host cell is a *Fusarium trichothecioides* cell.

16. The host cell of claim 1 in which the *Fusarium* host cell is a *Fusarium venenatum* cell.

17. The host cell of claim 1 in which the *Fusarium* host cell is a *Fusarium venenatum* Nirenberg *sp. nov* cell.

18. The host cell of claim 1 in which the *Fusarium* host cell is a *Fusarium toruloseum* cell.

19. The host cell of claim 1 in which the *Fusarium* cell has the identifying characteristics of ATCC 20334.

20. A method for producing a heterologous protein which comprises

(a) culturing a non-toxic, non-toxicogenic, non-pathogenic recombinant *Fusarium* host cell according to claim 1; and

(b) isolating the protein.

ABSTRACT

The present invention relates non-toxic, non-toxigenic, non-pathogenic recombinant *Fusarium* host cells of the section Discolor or a teleomorph or synonym thereof, comprising a nucleic acid sequence encoding a heterologous protein operably linked to a promoter.

100 101 102 103 104 105 106 107 108 109 110 111 112 113 114 115 116 117 118 119 120 121 122 123 124 125 126 127 128 129 130 131 132 133 134 135 136 137 138 139 140 141 142 143 144 145 146 147 148 149 150 151 152 153 154 155 156 157 158 159 160 161 162 163 164 165 166 167 168 169 170 171 172 173 174 175 176 177 178 179 180 181 182 183 184 185 186 187 188 189 190 191 192 193 194 195 196 197 198 199 200 201 202 203 204 205 206 207 208 209 210 211 212 213 214 215 216 217 218 219 220 221 222 223 224 225 226 227 228 229 230 231 232 233 234 235 236 237 238 239 240 241 242 243 244 245 246 247 248 249 250 251 252 253 254 255 256 257 258 259 260 261 262 263 264 265 266 267 268 269 270 271 272 273 274 275 276 277 278 279 280 281 282 283 284 285 286 287 288 289 290 291 292 293 294 295 296 297 298 299 300 301 302 303 304 305 306 307 308 309 310 311 312 313 314 315 316 317 318 319 320 321 322 323 324 325 326 327 328 329 330 331 332 333 334 335 336 337 338 339 340 341 342 343 344 345 346 347 348 349 350 351 352 353 354 355 356 357 358 359 360 361 362 363 364 365 366 367 368 369 370 371 372 373 374 375 376 377 378 379 380 381 382 383 384 385 386 387 388 389 390 391 392 393 394 395 396 397 398 399 400 401 402 403 404 405 406 407 408 409 410 411 412 413 414 415 416 417 418 419 420 421 422 423 424 425 426 427 428 429 430 431 432 433 434 435 436 437 438 439 440 441 442 443 444 445 446 447 448 449 450 451 452 453 454 455 456 457 458 459 460 461 462 463 464 465 466 467 468 469 470 471 472 473 474 475 476 477 478 479 480 481 482 483 484 485 486 487 488 489 490 491 492 493 494 495 496 497 498 499 500 501 502 503 504 505 506 507 508 509 510 511 512 513 514 515 516 517 518 519 520 521 522 523 524 525 526 527 528 529 530 531 532 533 534 535 536 537 538 539 540 541 542 543 544 545 546 547 548 549 550 551 552 553 554 555 556 557 558 559 560 561 562 563 564 565 566 567 568 569 570 571 572 573 574 575 576 577 578 579 580 581 582 583 584 585 586 587 588 589 590 591 592 593 594 595 596 597 598 599 600 601 602 603 604 605 606 607 608 609 610 611 612 613 614 615 616 617 618 619 620 621 622 623 624 625 626 627 628 629 630 631 632 633 634 635 636 637 638 639 640 641 642 643 644 645 646 647 648 649 650 651 652 653 654 655 656 657 658 659 660 661 662 663 664 665 666 667 668 669 670 671 672 673 674 675 676 677 678 679 680 681 682 683 684 685 686 687 688 689 690 691 692 693 694 695 696 697 698 699 700 701 702 703 704 705 706 707 708 709 710 711 712 713 714 715 716 717 718 719 720 721 722 723 724 725 726 727 728 729 730 731 732 733 734 735 736 737 738 739 740 741 742 743 744 745 746 747 748 749 750 751 752 753 754 755 756 757 758 759 760 761 762 763 764 765 766 767 768 769 770 771 772 773 774 775 776 777 778 779 780 781 782 783 784 785 786 787 788 789 790 791 792 793 794 795 796 797 798 799 800 801 802 803 804 805 806 807 808 809 810 811 812 813 814 815 816 817 818 819 820 821 822 823 824 825 826 827 828 829 830 831 832 833 834 835 836 837 838 839 840 841 842 843 844 845 846 847 848 849 850 851 852 853 854 855 856 857 858 859 860 861 862 863 864 865 866 867 868 869 870 871 872 873 874 875 876 877 878 879 880 881 882 883 884 885 886 887 888 889 890 891 892 893 894 895 896 897 898 899 900 901 902 903 904 905 906 907 908 909 910 911 912 913 914 915 916 917 918 919 920 921 922 923 924 925 926 927 928 929 930 931 932 933 934 935 936 937 938 939 940 941 942 943 944 945 946 947 948 949 950 951 952 953 954 955 956 957 958 959 960 961 962 963 964 965 966 967 968 969 970 971 972 973 974 975 976 977 978 979 980 981 982 983 984 985 986 987 988 989 990 991 992 993 994 995 996 997 998 999 1000

Figure 1

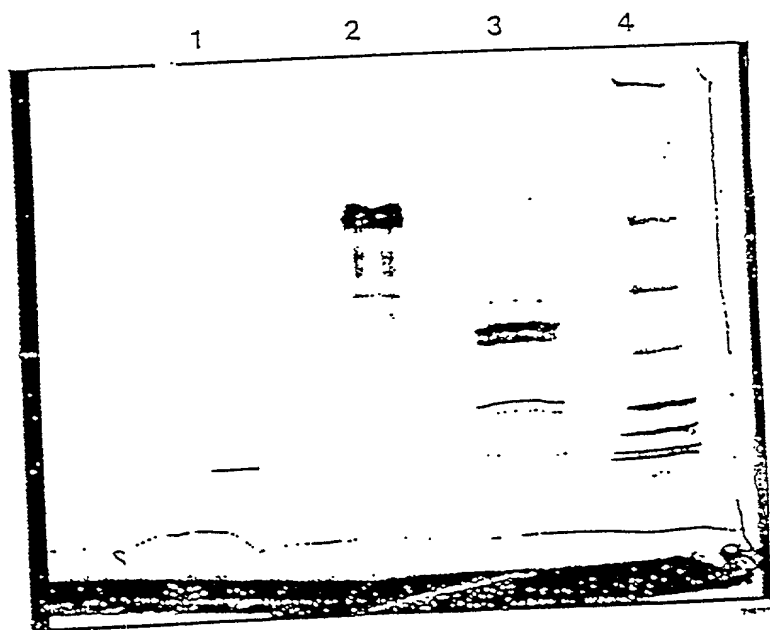


Fig -re .2

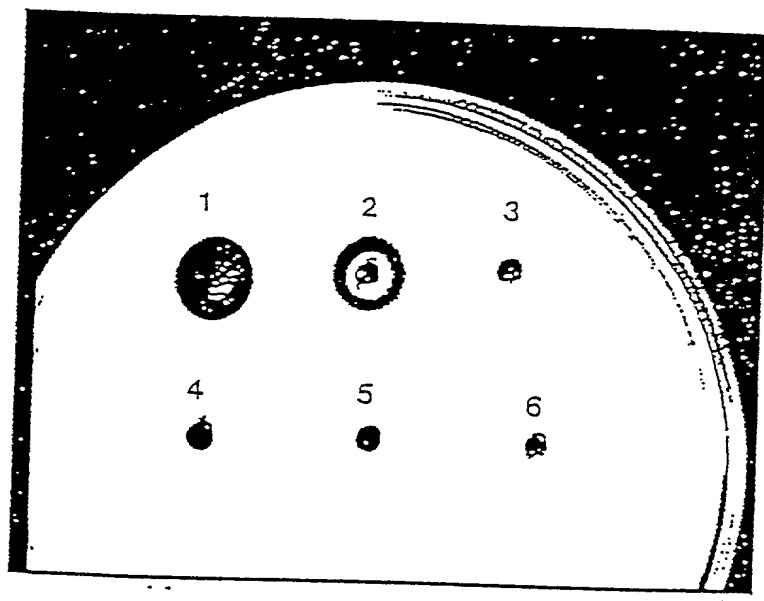
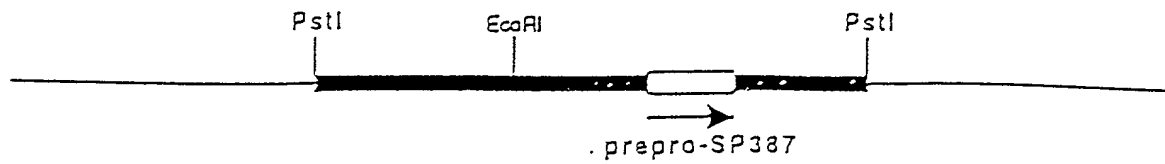
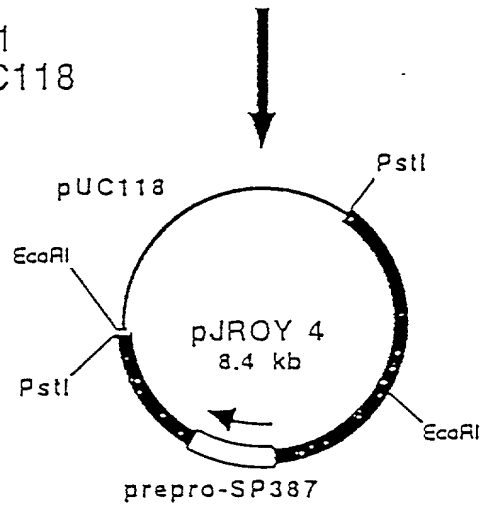


Figure 3

F. oxysporum DNA



Subcloning of PstI
fragment into pUC118



Subcloning of EcoRI
fragment into pToC90

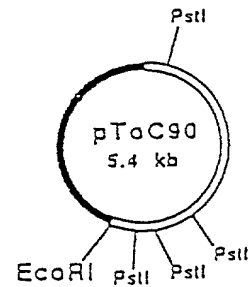
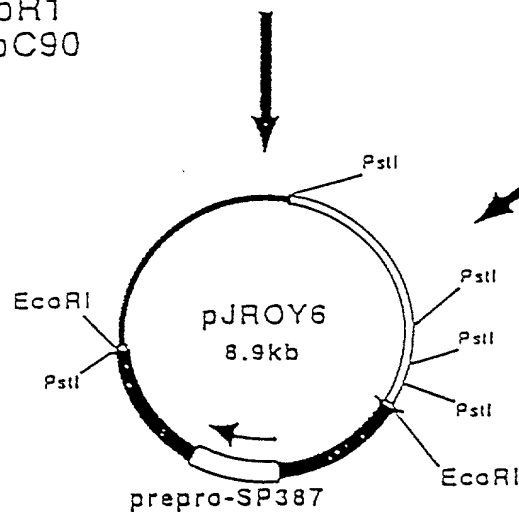


Figure 4

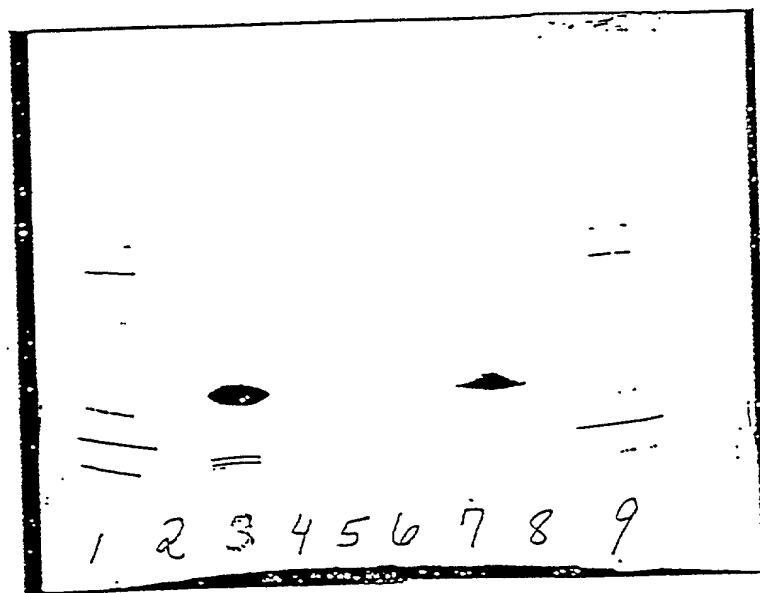


Figure 5

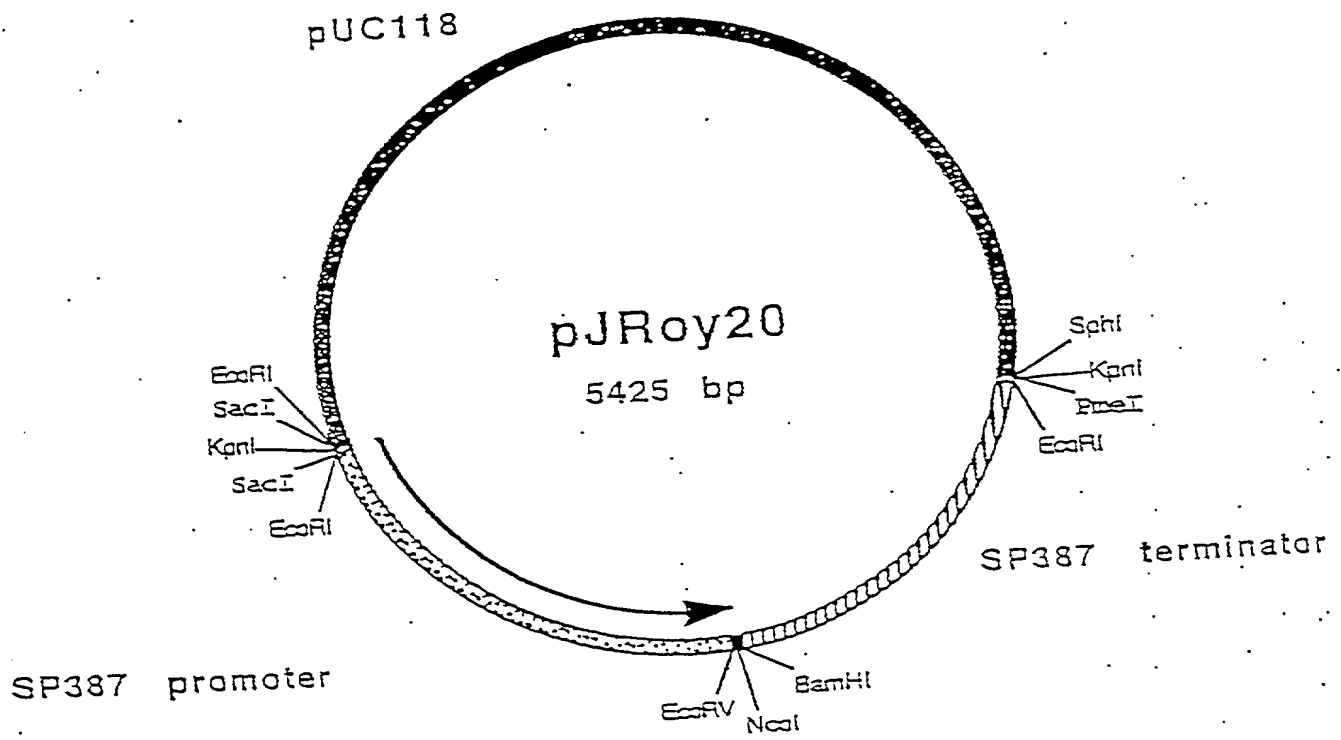


Figure 6

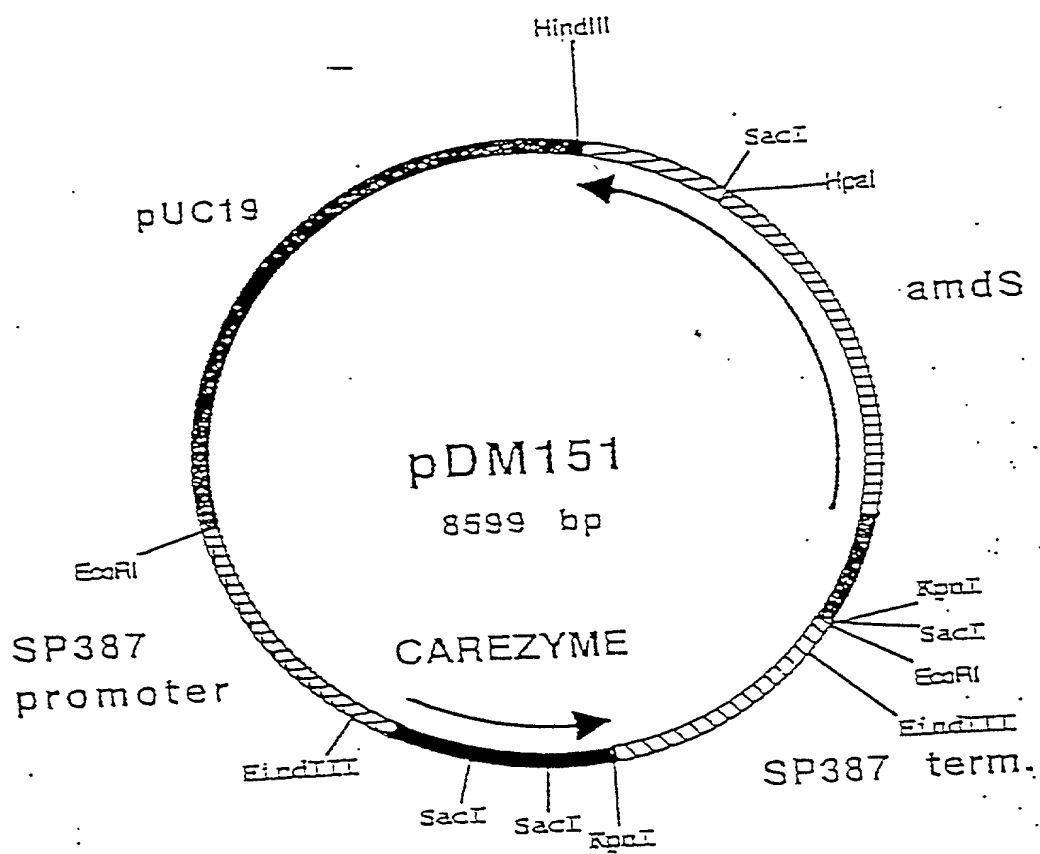


Figure 7

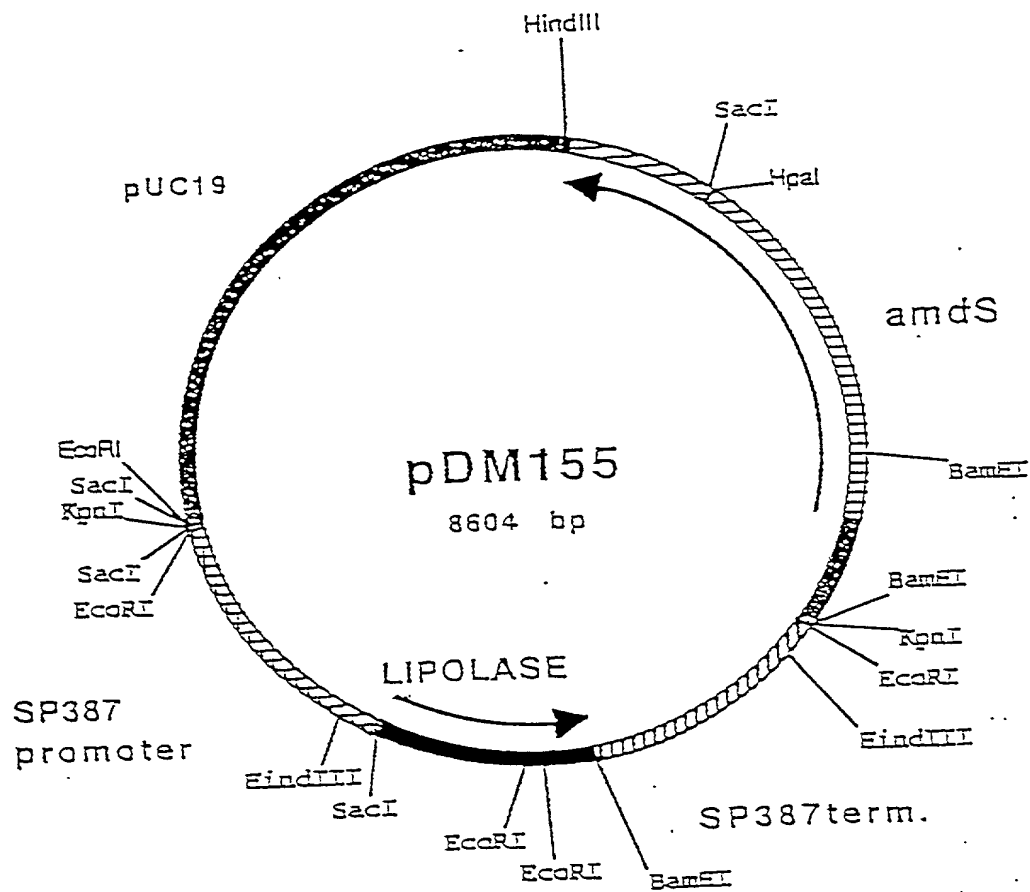


Figure 8A

Carezyme Expression in *F. graminearum*

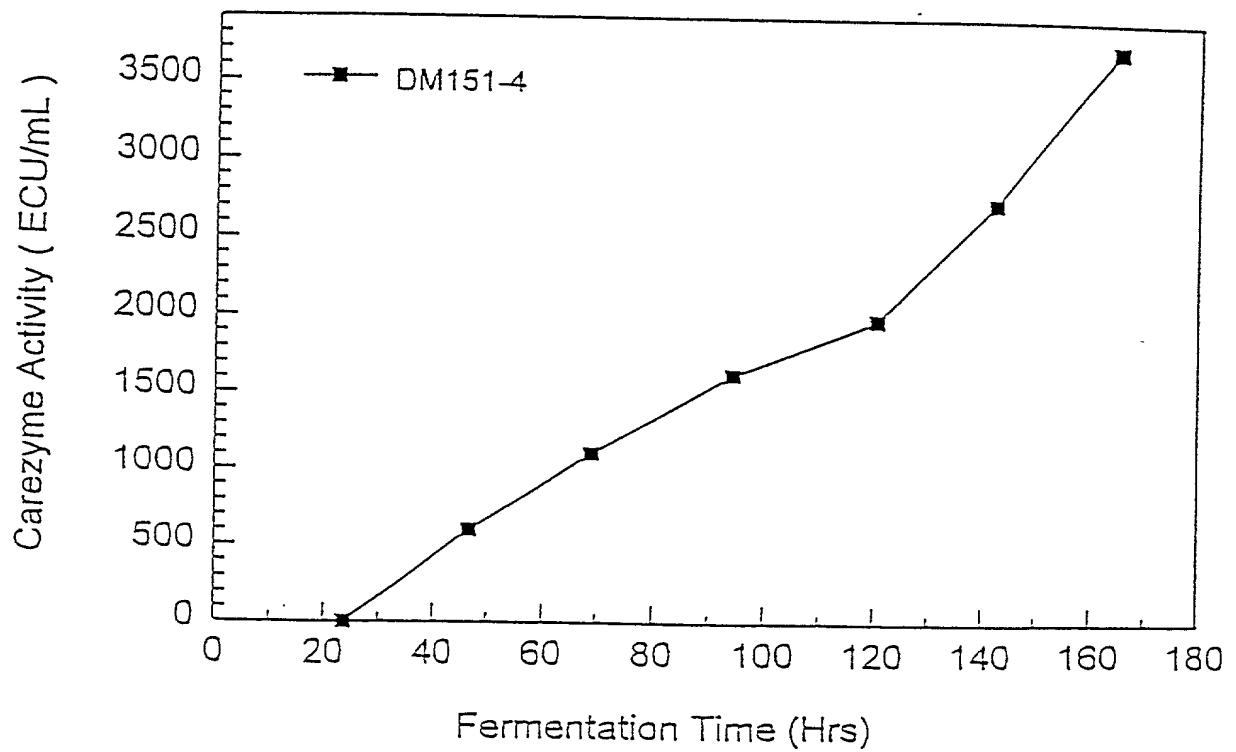


Figure 8B

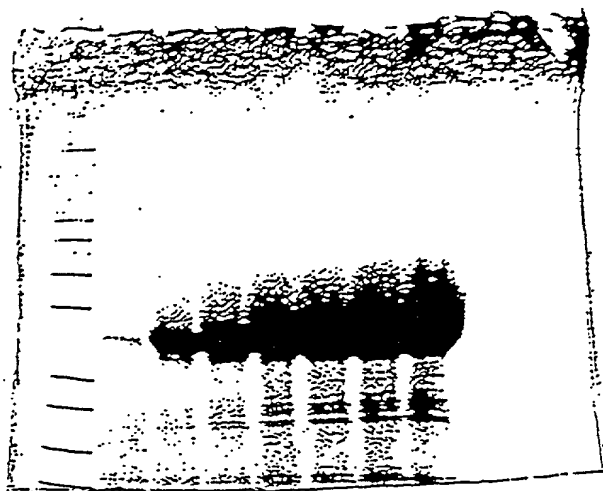


Figure 9.A

Lipolase in *F. graminearum*

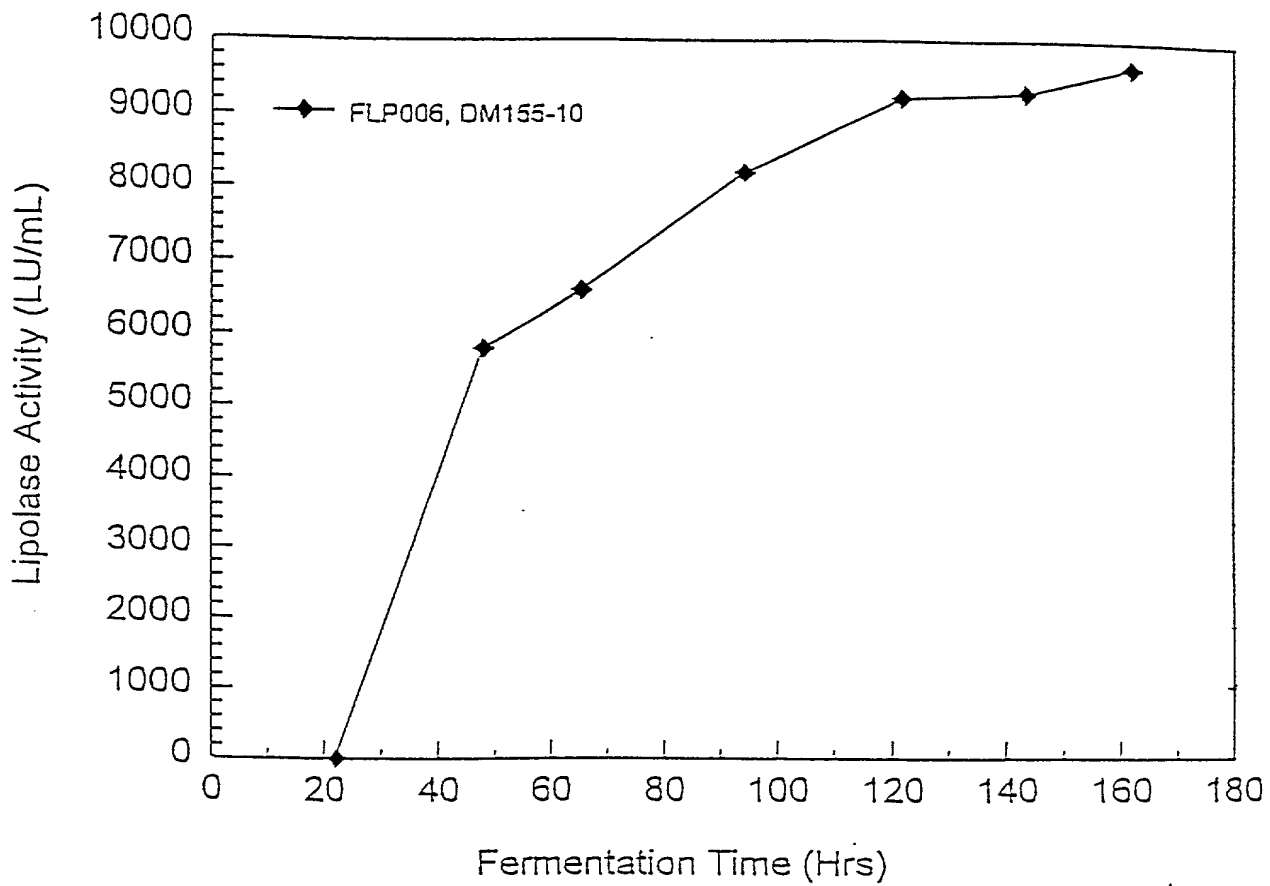


Figure 9B

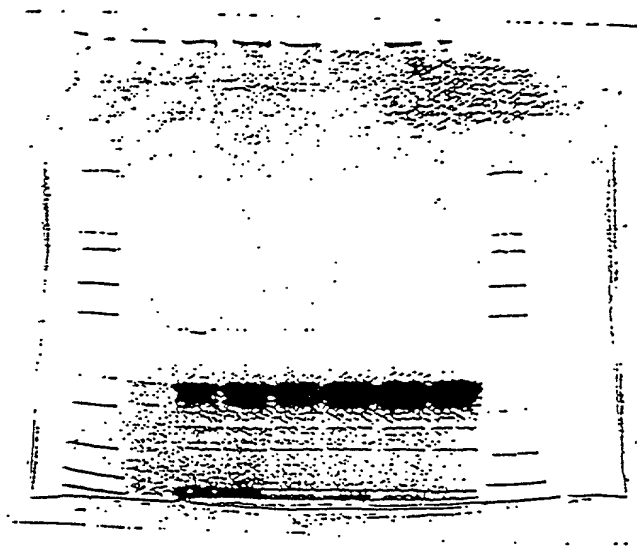


Figure 10

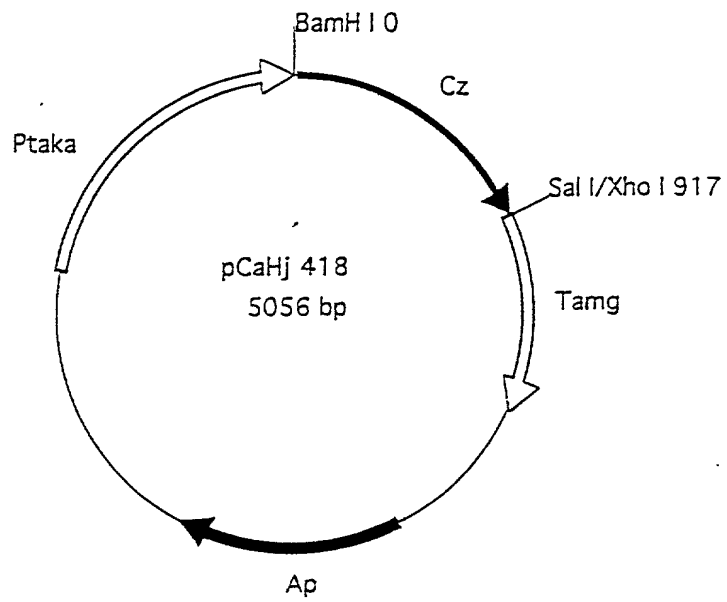


Figure 1

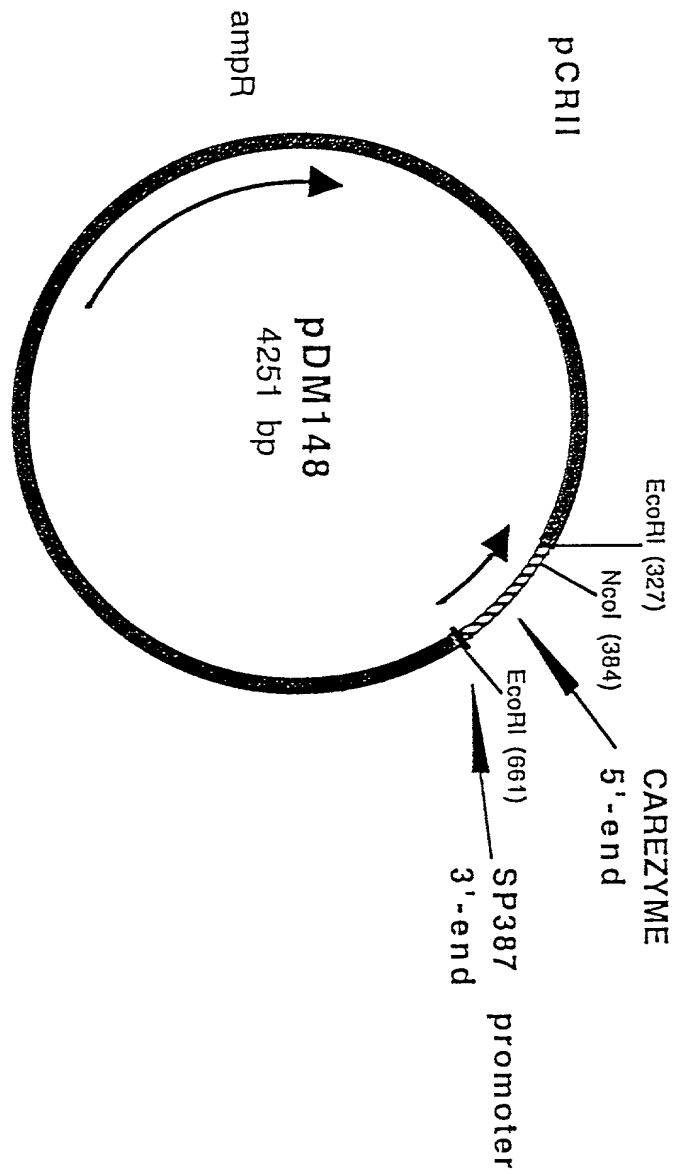


Figure 12

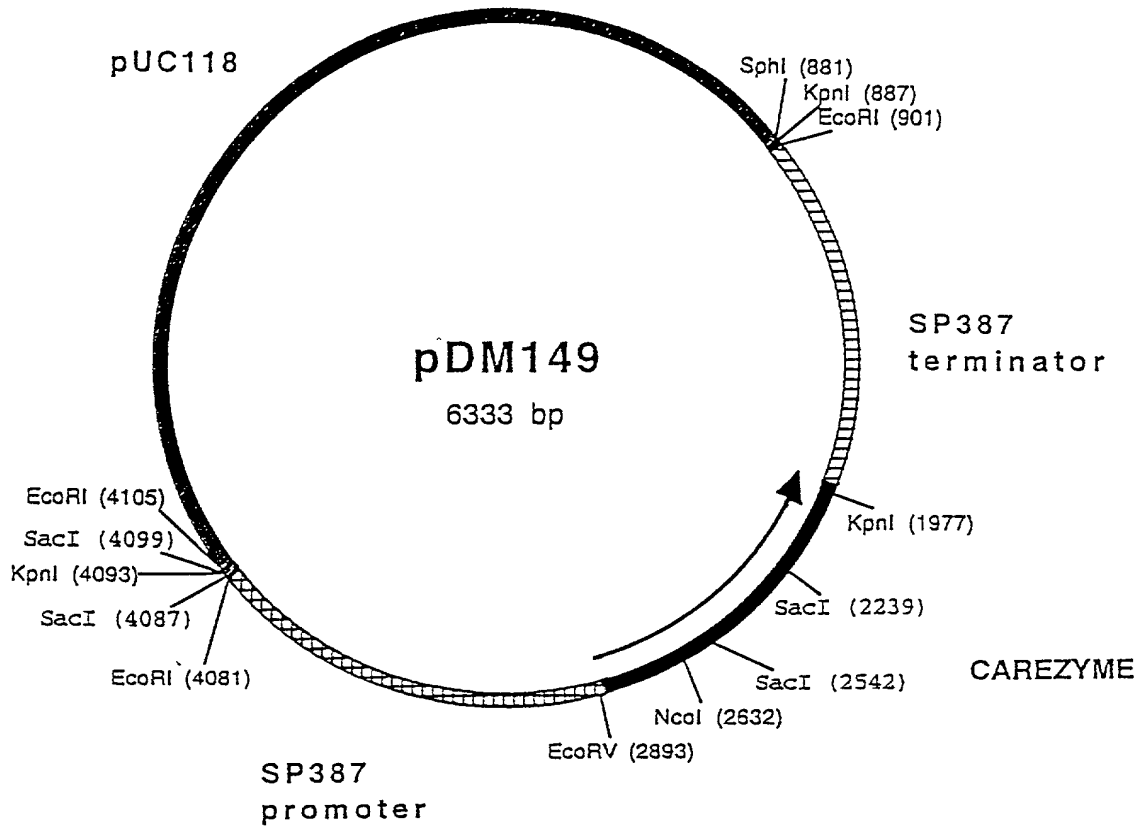


Figure 1. The effect of the number of iterations on the accuracy of the proposed algorithm. The accuracy is measured by the relative error of the solution. The relative error is defined as $\|u - u_h\|_2 / \|u\|_2$, where u is the exact solution and u_h is the numerical solution. The relative error is plotted against the number of iterations. The relative error decreases as the number of iterations increases. The relative error is approximately 10^{-6} after 100 iterations.

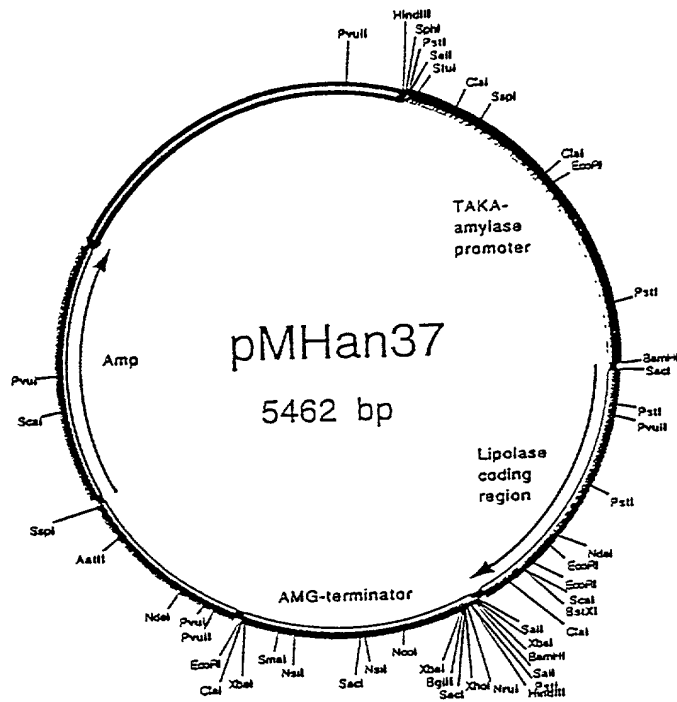
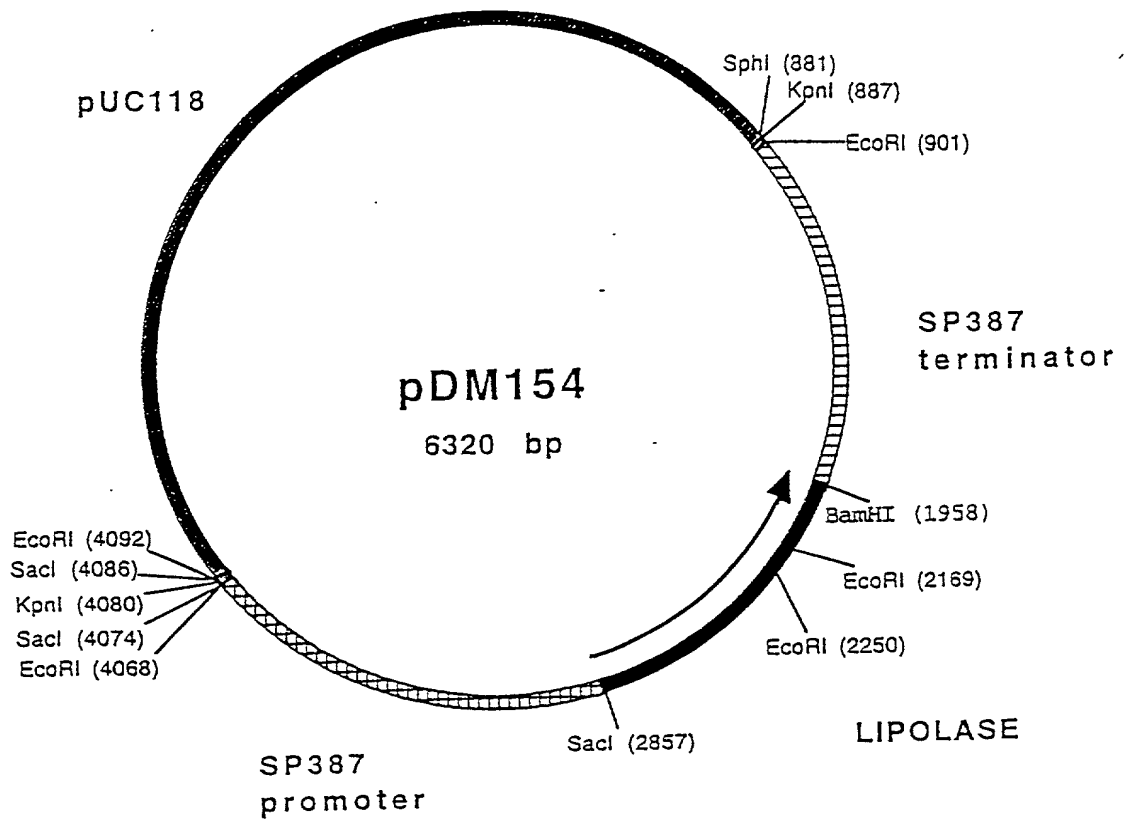


figure 14



COMBINED DECLARATION FOR PATENT APPLICATION AND POWER OF ATTORNEY
(Includes Reference to PCT International Applications)

Attorney's Docket Number
4216.240-US

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

Non-Toxic, Non-Toxicogenic, Non-Pathogenic Fusarium Expression System

the specification of which (check only one item below):

☐ is attached hereto

☒ was filed as United States application

Serial No. to be assigned

on March 13, 1997

and was amended

on _____

☐ was filed as PCT international application

Number _____

on _____

and was amended under PCT Article 19

on _____

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, §1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, §119 of any foreign application(s) for patent or inventor's certificate or of any PCT international application(s) designating at least one country other than the United States of America listed below and have also identified below any foreign application(s) for patent or inventor's certificate or any PCT international application(s) designating at least one country other than the United States of America filed by me on the same subject matter having a filing date before that of the application(s) of which priority is claimed:

PRIOR FOREIGN/PCT APPLICATION(S) AND ANY PRIORITY CLAIMS UNDER 35 U.S.C. 119:

COUNTRY (if PCT, indicate "PCT")	APPLICATION NUMBER	DATE OF FILING (day, month, year)	PRIORITY CLAIMED UNDER 35 USC 119
			<input type="checkbox"/> YES <input type="checkbox"/> NO
			<input type="checkbox"/> YES <input type="checkbox"/> NO
			<input type="checkbox"/> YES <input type="checkbox"/> NO
			<input type="checkbox"/> YES <input type="checkbox"/> NO
			<input type="checkbox"/> YES <input type="checkbox"/> NO
			<input type="checkbox"/> YES <input type="checkbox"/> NO

COMBINED DECLARATION FOR PATENT APPLICATION AND POWER OF ATTORNEY
(Includes Reference to PCT International Applications)

Attorney's Docket Number:
4216.240-US

I hereby claim the benefit under Title 35, United States Code §120 of any United States application(s) or PCT international application(s) designating the United States of America that is/are listed below and, insofar as the subject matter of each of the claims of this applications is not disclosed in that/those prior application(s) in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, §1.56(a) which occurred between the filing date of the prior application(s) and the national or PCT international filing date of this application:

PRIOR U.S. APPLICATIONS OR PCT INTERNATIONAL APPLICATIONS DESIGNATING THE U.S. FOR BENEFIT
UNDER 35 U.S.C. 120:

U.S. APPLICATIONS		STATUS (Check one)		
U.S. APPLICATION NUMBER	U.S. FILING DATE	Patented	Pending	Abandoned
08/269,449	June 30, 1994		x	
08/404,678	March 15, 1995		x	
08/726,105	October 4, 1996		x	

PCT APPLICATIONS DESIGNATING THE U.S.

APPLICATION NO.	FILING DATE	US SERIAL NUMBERS ASSIGNED (if any)			

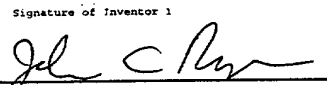
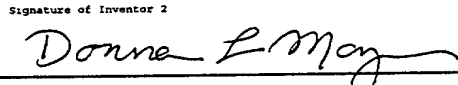
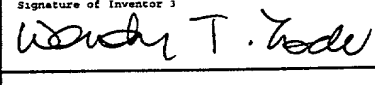
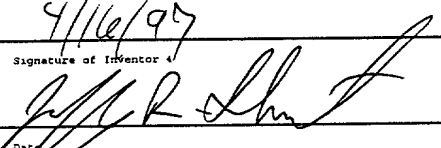
POWER OF ATTORNEY: As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith.
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Reg. No. 30,335 Reg. No. 33,728 Reg. No. 34,086 Reg. No. 38,711 Reg. No. 35,127

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COMBINED DECLARATION FOR PATENT APPLICATION AND POWER OF ATTORNEY (Includes Reference to PCT International Applications)				Attorney's Docket Number: 4216.240-US	
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<p>I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.</p>					
Signature of Inventor 1		Signature of Inventor 2		Signature of Inventor 3	
					
Date 4/16/97		Date 4.15.97		Date 4.15.97	
Signature of Inventor 4		Signature of Inventor 5		Signature of Inventor 6	
					
Date 4/15/97		Date		Date	
Signature of Inventor 7		Signature of Inventor 8		Signature of Inventor 9	
Date		Date		Date	